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(54) Title: ANT-1 AS DRUG TARGET

(57) Abstract: The present invention refers to the use of adenine nucleotide translocase-1 (ANT-1), a central component of the permeability transition pore in mitochondria, as a drug target, particularly for the treatment of dilated cardiomyopathy. Further, an assay for the detection of pharmacologically active substances is disclosed which inhibit ANT-1 activity. In addition, ANT-1 expression is claimed as a diagnostic marker for dilated cardiomyopathy.

ANT-1 as Drug Target

Description

The present invention refers to the use of adenine nucleotide translocase-1 (ANT-1), a central component of the permeability transition pore in mitochondria, as a drug target, particularly for the treatment of dilated cardiomyopathy. Further, an assay for the detection of pharmacologically active substances is disclosed which inhibit ANT-1 activity. In addition, ANT-1 expression is claimed as a diagnostic marker for dilated cardiomyopathy.

Apoptosis is a form of cell death that plays a role in development, tissue homeostasis and disease (White, 1996). Its induction must be tightly regulated. Otherwise, serious consequences may follow: A hyperactive apoptosis induction might lead to degenerative diseases like Alzheimer's disease (Loo et al., 1993); a reduced activity can contribute to the multistep process of tumorigenesis, since tumor cells are exposed to multiple proapoptotic stimuli (McGill, 1997). The induction of apoptosis is therefore governed by an elaborate array of checks and balances in the cell. Eventually, a family of cysteine proteases, the so-called caspases, is activated (Salvesen and Dixit, 1997). These enzymes can cleave specific substrates in the cell which leads to the typical apoptotic phenotype and the self-destruction of the cell.

The first indication that mitochondria play a role in apoptosis induction was the observation that an in vitro system for apoptosis induction required the presence of mitochondria (Newmeyer et al., 1994). The permeability transition pore (PT) complex (Zoratti and Szabo, 1995), a protein aggregate that resides in contact sites of the inner and outer mitochondrial membrane, was subsequently identified as being responsible for apoptosis induction

(Petit et al., 1995; Zamzami et al., 1995b). Multiple pharmacological stimuli have been shown to activate this complex by as yet unknown means (Fulda et al., 1998; Petit et al., 1995; Zamzami et al., 1998). The activated complex leads to the collapse of the potential over the inner mitochondrial membrane ($\Delta\Psi_m$), swelling of mitochondria and the generation of oxygen radicals (Vander Heiden et al., 1997; Zamzami et al., 1995a). The subsequent release of apoptogenic caspases (Susin et al., 1999a) and a putative oxidoreductase (Susin et al., 1999b) aids in apoptosis induction.

Many genes involved in apoptosis have the dominant capacity to induce cell death upon overexpression. This feature is even conserved across species (McCarthy and Dixit, 1998). It might be explained by the fact that the overexpressed proteins engage in protein-protein contacts and can thereby create the signal for apoptosis (Yang et al., 1998). Consequently, we have recently developed a screen for dominant, apoptosis-inducing genes (Grimm and Leder, 1997). The screen is based on the iterative transfection of small plasmid pools of a normalized cDNA library into mammalian cells and the morphological determination of apoptosis induction.

Here we describe the isolation of adenine nucleotide translocase-1 (ANT-1), a central component of the permeability transition pore, using such a screen. ANT-1 has recently been shown to be required for apoptosis mediated by Bax, another component of the PT complex (Marzo et al., 1998a). Conversely, ANT-1 can induce apoptosis in a Bax-dependent manner. For this, ANT-1 must be activated pharmacologically by the chemical atractyloside which arrests ANT-1 in a specific conformation and causes PT pore opening (Marzo et al., 1998a). In our experiments with ANT-1, however, such a secondary signal is not required for apoptosis induction. ANT-1's apoptosis activity does not seem to depend on its known function for ADP/ATP exchange because several transport inactive mutants could still lead to cell death. Surprisingly, we found that a very homologous protein, the ANT-2 transporter, was inactive for apoptosis

induction. Furthermore, ANT-1 could not elicit a form of cell death in yeast. This is in contrast to Bax that directly interacts with ANT-1 and can dominantly induce cell death in yeast. This result suggests that ANT-1-mediated apoptosis induction depends on protein-protein interactions that are specific for mammalian cells and is independent of Bax-mediated apoptosis. Since ANT-1 is activated by overexpression for apoptosis induction, it is interesting to note that this gene is already highly expressed in mitochondria. This suggests that in a normal cell it must be kept inactive by other proteins of the PT pore which implies important stoichiometric correlations between the various components of this complex. Consistent with this, we found that cyclophilin D, another component of the PT pore can repress ANT-1-induced apoptosis. Furthermore, our data help to explain the observed apoptosis induction in dilated cardiomyopathy (DCM), a degenerative disease of the heart muscle which is marked by a dramatic increase of the expression level of ANT-1 and by excessive apoptosis induction.

These findings are up to now the most direct genetic evidence that the PT pore can signal apoptosis and may lead to the molecular elucidation of how this complex can be activated for apoptosis induction.

A first aspect of the present invention is a method for the inhibition of apoptosis comprising the step of contacting a cell which is susceptible to apoptosis with an effective amount of a substance capable of inhibiting the activity of adenine nucleotide translocase-1 (ANT-1), particularly the apoptosis-inducing activity of ANT-1.

The cell is preferably a vertebrate cell, more preferably a mammalian cell and, still more preferably, a human cell, e.g. a human myocyte.

The inhibition may effect an apoptosis-inducing signal transduction pathway, said pathway being activated by ANT-1. The activity of ANT-1

may be inhibited on the nucleic acid level and/or on the protein level. Inhibition on the nucleic acid level may be effected by reducing ANT-1 gene expression, e.g. by using antisense-oligonucleotides which may inhibit the ANT-1 gene transcription, by using ribozymes capable of cleaving ANT-1 transcripts or by inhibiting the activity of the endogenous promoter, e.g. via ANT-1-specific transcription factors. The reduction of ANT-1 expression may also be achieved by influencing the activity of proteins that act upstream of these transcription factors, e.g. such as protein kinases and phosphatases. Inhibition on the protein level may be effected by anti-ANT-1 antibodies, by ANT-1 regulatory proteins, e.g. by proteins occurring in permeability transition pores such as cyclophilin D or by low molecular weight substances which are capable of inhibiting the apoptotic or proapoptotic activity of ANT-1.

A further subject matter of the present invention is a method for the treatment of diseases associated with excessive apoptosis, comprising the step of administering to a subject in need thereof a pharmaceutically effective amount of a substance capable of inhibiting the activity of adenine nucleotide translocase-1 (ANT-1), particularly the apoptosis-inducing activity of ANT-1. Examples of diseases which may be treated by administering ANT-1 antagonists are dilated cardiomyopathy and other human pathogenic disorders associated with excessive apoptosis such as degenerative diseases. More preferably, the disease is dilated cardiomyopathy.

The ANT-1 antagonist may be administered as a pharmaceutical composition comprising the active agent, optionally together with pharmaceutically acceptable diluents, carriers or adjuvants. The pharmaceutical composition may be suitable for oral, parenteral, e.g. intradermal, intravenous or intramuscular, rectal, nasal and topical applications. The composition may be an injectable solution, ointment, cream or spray. Further, the composition may have retardation properties, e.g. showing a delayed release of the active agent.

The dosage of the active agent depends on the specific compound being administered, the type and the severity of the disease. For example, a dosage from 0.01 mg to 100 mg per day and per kg body weight of the active agent is suitable.

Still a further aspect of the present invention is a method for identifying substances suitable for apoptosis inhibition, comprising the step of determining the capability of a test substance to inhibit the activity of ANT-1, particularly the apoptosis-inducing activity of ANT-1. Preferably, the capability of a test substance to bind ANT-1 or a domain thereof may be determined. Particularly, the N-terminal domain of ANT-1 comprising amino acids 1-150, preferably amino acids 1-200, is suitable for determining the binding of test substances. Alternatively, the capability of test substances to inhibit the binding of ANT-1 to natural binding partners thereof such as cyclophilin D may be determined.

The identification of ANT-1 antagonists may be carried out as a high-throughput assay which preferably comprises a parallel determination of at least 24 and, more preferably, at least 96 test compounds. The assay may be carried out as a cell-based assay, wherein a test system is used comprising ANT-1-containing cell fractions or ANT-1-containing whole cells. Alternatively, the assay may be carried out as a molecular-based assay comprising a substantially purified and isolated protein selected from ANT-1 or a domain thereof, particularly an N-terminal domain thereof. The substantially purified ANT-1 protein may be a recombinant ANT-1 protein which may a soluble protein (by deletion of membrane anchor domains) or a membrane-bound protein.

In a cell-based assay the determining step may comprise the measurement of apoptosis induction. Apoptosis induction may be measured by a parameter which is associated with apoptosis such as DNA fragmentation, caspase activation or characteristic alterations in cell morphology or other

qualitative and/or quantitative methods of apoptosis measurement known in the field. In molecular-based assay systems the determination may comprise the measurement of protein-protein interactions, e.g. the interaction of ANT-1 with other proteins, or direct measurement of the interaction of test compounds with ANT-1.

As the invention describes upregulated ANT-1 as a drug target in dilated cardiomyopathy or other degenerative diseases, a further aspect of this invention is the examination of the ANT-1 expression level in patients as a diagnostic marker. The ANT-1 expression can be detected and quantified by conventional methods, e.g. on the RNA level by hybridization and/or amplification procedures or on the protein level by antibody and/or activity assays. Thus, the invention relates to a method for the diagnosis of a degenerative disease or a predispositon therefor comprising detecting the ANT-1 expression in a sample from tissue and/or body fluids of a subject to be tested, wherein elevated ANT-1 expression is indicative for a degenerative disease or a predisposition therefor.

The present invention is further illustrated by the following Figures and Examples.

Description of Figures

Fig. 1. Adenine nucleotide translocase-1 (ANT-1) expression leads to phenotypic apoptosis induction. The empty vector or an expression plasmid for ANT-1 were transiently transfected into 293T cells. After 16 hours, phase contrast pictures were taken at a 200-fold magnification.

Fig. 2. Effect of ANT-1 expression on mitochondria. (**A**) ANT-1 overexpression leads to the collapse of the inner mitochondrial membrane potential. HeLa cells were transfected with an expression vector for GFP (2 μg) together with a control vector ("Vector"; 2 μg) or a plasmid for

ANT-1 ("ANT-1"; 2 μg). After 16 hours, the cells were treated with CMXRos which stains mitochondria with an intact membrane potential. Subsequently, phase contrast and fluorescence microscopy pictures for GFP and CMXRos activity were taken. (B) ANT-1 induces the release of cytochrome c from mitochondria. 293T cells were transfected with a control vector or an expression vector for ANT-1. Cytoplasmic extracts were prepared and assayed for the presence of cytochrome c by a western blot. Molecular weight standards are given on the left. Equal loading of the gel is indicated by an upper unspecific band with equal intensity.

Fig. 3. Effect of ANT-1 on protein and DNA degradation. (**A**) ANT-1 leads to the degradation of poly-ADP-ribose polymerase (PARP). 293T cells were transfected with an empty control vector, an expression vector for the "death domain" protein RIP or with ANT-1. 16 hours later, nuclear extracts of the transfected cells were prepared and investigated for the status of PARP in a western blot. The generated PARP fragment is indicated by an arrow. An unspecific signal (o) served as an internal control for equal loading of the gel. (**B**) Inhibition of ANT-1 apoptosis by a specific inhibitor for caspases. ANT- 1 (1 μg) and a control vector (10 μg) or an expression vector (10 μg) for the caspase inhibitor p35 from baculovirus were cotransfected, and the specific apoptosis induction was determined by FACS analysis. The means and the standard deviations are indicated (n = 3) (**C**) ANT-1 induces internucleosomal DNA cleavage. 293T cells were transfected with an empty vector or with an expression construct for ANT-1. Low molecular weight DNA was isolated from transfected cells, separated on a 2% agarose gel and stained with ethidium bromide.

Fig. 4. Mutational analysis of ANT-1's apoptosis activity (**A**) Cell death induction by point mutants of ANT-1. Wild type ANT-1 (ANT-1 WT) and six point mutants that have been shown to be deficient for ADP/ATP transport were transfected into 293T cells. The WT amino acid, its position as well as the mutated residue are given for each mutant construct. After 16

hours, apoptosis induction by the various constructs was measured by an ELISA for internucleosomal DNA fragments. The DNA fragmentation as percentage of control is given as an index for apoptosis induction. The means and the standard deviations are indicated (n = 3) (**B**) Apoptosis activity of ANT-1 deletion mutants. Wild type ANT-1 (1 μg) and three C-terminal deletion mutants (1 μg) were transiently transfected into 293T cells. The last C-terminal residue of each deletion mutant is indicated. After 16 hours, the constructs were tested for apoptosis induction by a specific ELISA as described in A.

Fig. 5. Effect of ANT-1 and ANT-2 on transfected cells. (**A**) Comparison between the apoptosis capacities of ANT-1 and ANT-2. Expression plasmids for ANT-1 (1 μg) and ANT-2 (1 μg) were transfected in 293T cells. After 16 hours apoptosis induction in transfected cells was measured by FACS analysis of sub-G1 positive cells. The specific apoptosis induction above background is shown as percentage of apoptotic cells relative to all transfected cells. The means and the standard deviations are indicated (n = 3). (**B**) Expression and localization of ANT-1 and ANT-2. 293T cells were transfected with a control vector or expression vectors for HA-tagged ANT-1 and ANT-2. Mitochondrial extracts of transfected cells were prepared and investigated for the presence of the proteins with an anti-HA antibody in a western blot. Equal loading of the gel was verified by two unspecific upper bands.

Fig. 6. Effect of Bax and ANT-1 expression on the growth of yeast cells. Bax and ANT-1 cloned in expression vectors under the control of a galactose and raffinose-inducible promoter and the empty control vector were introduced into yeast cells. $4{,}5 \times 10^4$ yeast cells of three independent clones each were plated on glucose (glu)- or galactose and raffinose (gal/raf)-containing agar. The growth of the yeast cells was monitored 36 hours later.

Fig. 7. Inhibition of ANT-1 induced apoptosis. (A) ANT-1-induced apoptosis can be repressed by bongkrekic acid, a specific inhibitor of the PT pore. An expression vector for ANT-1 (1 μg) was transfected into plates with 293T cells. One group of plates was left untreated, the other was supplied with 50 μM bongkrekic acid ("BA") for 16 hours after the transfection. Apoptosis was quantified as in figure 5. (B) ANT-1 apoptosis can be repressed by cyclophilin D, another component of the permeability transition pore. ANT-1 (1 μg) and an expression plasmid (10 μg) for cyclophilin D ("Cyclo D") or a control vector (10 μg) were transfected into 293T cells, and apoptosis was determined as described in figure 5.

Examples

1.Materials and methods

1.1 Plasmids

The point mutants of ANT-1 were engineered by recombinant PCR using suitable primers. For the PCR reaction, Pwo, a thermostabie polymerase with proof-reading function (Roche Diagnostics, Mannheim, Germany) was employed. All amino acid changes were verified by sequencing. Deletion mutagenesis of ANT-1 was achieved with PCR using the same enzyme. ANT-2 was amplified from a mouse kidney cDNA library, cyclophilin D from a 293T library, both by using specific primers. The correct sequence was verified by sequencing. The eukaryotic expression vector for baculovirus p35 has been described (Clem and Miller, 1994).

1.2 Immuno Blotting

For detecting cytochrome c, 293T cells were transfected with an expression vector for ANT-1. After 18 hours the cells were harvested and cytoplasmic extracts isolated as described (Ferrari et al., 1998). Protein samples of 30

µg were loaded on SDS-polyacrylamide gels (10%) and then electrophoretically transferred onto PVDF-membranes (Amersham, Braunschweig, Germany). Western blots were probed with a monoclonal cytochrome c antibody (Pharmingen, San Diego, CA) and anti-mouse-horseradish-peroxidase conjugated antibody prior to ECL-based detection (Amersham). For the poly-ADP-ribose polymerase (PARP) western blot, nuclear fractions were obtained by differential centrifugation as described previously (Schreiber et al., 1989). Aliquots of 50 µg protein were subjected to SDS-PAGE (8% polyacrylamide) and blotted onto PVDF-membranes. PARP and cleavage products were detected using a polyclonal serum directed against full length PARP (Roche Diagnostics), anti-rabbit-horseradish-peroxidase conjugated antibody (Amersham, Braunschweig) and the ECL-system. For the detection of HA-tagged ANT-isoforms, mitochondria were isolated by differential centrifugation as described previously (Brustovetsky and Klingenberg, 1996). 30 µg protein samples were separated on 10% polyacrylamide gels and blotted onto PVDF-membranes. Detection was performed using a mouse monoclonal antibody raised against the influenza virus HA-peptide (Roche Diagnostics, Mannheim, Germany) and the ECL-system (Amersham, Braunschweig).

1.3 Yeast methods

Standard yeast methods were applied (Ausubel et al., 1991). For the inducible expression of Bax and ANT-1, the cDNAs were subcloned into pYESTrp2 (Invitrogen) in which the B42-fusion moiety was removed.

1.4 Apoptosis detection

Low molecular weight DNA from apoptotic cells was isolated and detected as described (Grimm and Leder, 1997). Apoptosis induction was measured by an ELISA (Roche Diagnostics) that is specific for nucleosomal DNA fragments that are released during apoptosis. The recommendations of the

manufacturer were followed. Equal transfection efficiencies were monitored by cotransfecting an expression plasmid for GFP. Other apoptosis quantifications were performed by flow cytometry (Bitzer et al., 1999). A cotransfected GFP expression plasmid was used to assess the transfection efficiency. The apoptotic population was put in relation to the percentage of GFP-positive cells. The apoptotic background of the vector-control was subtracted to obtain the specific apoptosis induction. Each condition was tested in at least three independent experiments.

1.5 Mitochondrial membrane gradient detection

After transfecting an expression vector for ANT-1 together with a plasmid for GFP, cells were loaded with Mitotracker RedCMXRos (Molecular probes, OR, USA) according to the suggestions of the manufacturer. Images were documented using a Zeiss Axioplan fluorescence microscope (Zeiss Oberkochen, Germany). RedCMXRos fluorescence was excited at 546 nm and emission was imaged at >590 nm. GFP fluorescence was excited at 450-490 nm and emission was monitored at 515-565 nm.

2.Results

2.1 ANT-1 induces cell death

Using our screen for dominant, apoptosis-inducing genes, we isolated a cDNA whose expression elicited an especially fast and strong apoptosis response in cells. Sequencing revealed that the gene encoded ANT-1, the ADP/ATP translocator protein of the PT pore (Marzo et al., 1998b). ANT was active in a wide variety of cell types: every cell line tested so far underwent apoptosis after stimulation by ANT-1 (not shown). Figure 1 shows the phenotype of control- and ANT-1-transfected HEK 293T cells: whereas the vector-transfected cells displayed a normal morphology, ANT-1

expressing cells showed a constricted cytoplasm and blebbing of the plasma membrane.

Since ANT-1 is a component of the PT pore whose activation leads to a dissipation of the proton gradient $\Delta\Psi_m$ over the inner mitochondrial membrane (Marzo et al., 1998b), we tested ANT-1 transfected cells for this membrane gradient. To this end, we cotransfected ANT-1 and an expression vector for the green fluorescent protein (GFP) into HeLa cells. The membrane gradient in transfected and therefore GFP-positive cells was assayed with CMXRos, a dye whose fluorescence is dependent on an intact membrane potential. Figure 2A shows that ANT-1 expressing cells displayed a reduced mitochondrial fluorescence with this dye. This differential staining is a very early feature of ANT-1-mediated apoptosis induction since it could be observed in cells which did not yet display an altered phenotype.

One of the consequences of mitochondrial damage is the release of cytochrome c from the inner mitochondrial membrane space (Kluck et al., 1997; Yang et al., 1997). Therefore, we assayed cytoplasm of ANT-1 transfected cells for the presence of cytochrome c. Using a western blot, we could detect this protein in the cytoplasmic fraction of ANT-1 transfected cells (figure 2B). The released cytochrome c can bind Apaf-1, a mammalian homolog of the C. elegans gene ced-4 which leads to the aggregation and activation of downstream caspases (Srinivasula et al., 1998). We therefore investigated the status of poly-ADP-ribose polymerase (PARP), one of the best-known caspase substrates, in ANT-1 transfected cells. A western blot for PARP (figure 3A) revealed that ANT-1 transfected cells degraded PARP and generated the expected protein fragments. Since we have also observed apoptosis induction in MCF-7 cells (data not shown), a cell line that does not express caspase-3 (Janicke et al., 1998), we conclude that ANT-1-induced apoptosis is not dependent on this particular isoenzyme.

Bax, a member of the Bcl-2 gene family, has recently been shown to interact directly with ANT-1 (Marzo et al., 1998a) and is known to induce apoptosis (Oltvai et al., 1993). However, caspase activation does not seem to be a necessary event for Bax-induced cell death (Xiang et al., 1996). Therefore, we wanted to know whether caspase activation is required for ANT-1 apoptosis induction. To address this, we cotransfected ANT-1 and an expression vector for p35, a broad-range caspase inhibitor from baculovirus (Clem and Miller. 1994). A quantitative apoptosis assay revealed a 6-fold reduction in apoptosis induction when p35 was cotransfected (figure 3B). Another substrate for caspases is ICAD, an inhibitor of the DNase CAD (Sakahira et al., 1998). Its degradation leads to the activation of CAD and the internucleosomal degradation of the DNA, a well-known biochemical sign of apoptosis. To investigate the status of the DNA, we transfected 293T cells with a control vector or ANT-1 and isolated the low molecular weight DNA. Only ANT-1 transfected cells displayed the typical DNA ladder (figure 3C). These results show that ANT-1 leads to all phenotypic and biochemical alterations associated with apoptosis.

2.2 The N-terminal half of ANT-1 is sufficient for apoptosis induction

ANT-1 was originally described as an ADP/ATP transporter (Riccio et al., 1975). We therefore theorized that overexpressed ANT-1 leads to an increased ADP/ATP exchange which results in a net-import of positive charges over the inner mitochondrial membrane, the subsequent collapse of the membrane potential and apoptosis induction. To assess whether the transporter activity of ANT-1 is required for its apoptosis induction, we generated six different point mutations all of which have been shown to impede ANT-1's activity to transport ADP and ATP (Muller et al., 1996; Muller et al., 1997). However, upon transfection into 293T cells we have not observed any difference relative to the wildtype (WT) in their potential to induce apoptosis as measured by the release of internucleosomal DNA

fragments (figure 4A). ANT-1 is a member of the mitochondrial carrier family which consists of proteins with three 100 residue repeats in which alternating regions of hydrophilic and hydrophobic residues exist. As integral membrane proteins they span the membrane six times, interrupted by short amphipathic helices that are likely to form the transport active moiety (Klingenberg and Nelson, 1994). To map the domains responsible for apoptosis induction, we have created various deletion mutants of ANT-1:ANT-1Δ 217 lacks the last transmembrane domain and the last amphipathic helix, ANT-1Δ 142 cuts the molecule in half and comprises only the first two transmembrane domains and the first amphipathic helix. ANT-1Δ 102 contains only the first membrane spanning domain. Figure 4B demonstrates that ANT-1Δ 217 is as efficient for apoptosis induction as WT ANT-1, whereas ANT-1Δ 142 revealed a 50% reduction of its apoptosis potential. The largest deletion, ANT-1Δ 102, was inactive for apoptosis induction.

2.3 ANT-2 is unable to induce apoptosis

The PT pore complex is supposed to function by the conversion of the specific ADP/ATP exchange transporter into an unselective pore (Zoratti and Szabo, 1995). However, since over half of the ANT-1 protein is dispensable for apoptosis induction, we wanted to know whether ANT-1 induces apoptosis by forming an unspecific pore on its own. We therefore assessed the specificity of apoptosis induction by ANT-1. To this end we made use of ANT-2, an over 90% identical gene to ANT-1 that is likewise ubiquitously expressed (Doerner et al., 1997). Transfection of ANT-2 did not lead to apoptosis induction as measured by FACS analysis (figure 5A). The correct mitochondrial localization and efficient expression of ANT-2 was verified by western blot (figure 5B).

2.4 Growth of yeast cells is not affected by ANT-1

Bax, a member of the Bcl-2 gene family, can induce a form of cell death by overexpression in yeast cells (Greenhalf et al., 1996; Jurgensmeier et al., 1997; Zha et al., 1996), probably by activating ANT-1 and the PT pore (Greenhalf et al., 1996; Marzo et al., 1998a). We therefore wanted to determine whether ANT-1 has the same capacity. To address this, we subcloned ANT-1 and Bax into a galactose and raffinose inducible yeast vector. The induction of these genes on suitable agar plates revealed that Bax led to a reduction in the growth of these cells, indicating cell death. In contrast, ANT-1 expressing yeast cells were completely unaffected and grew as well as on the control plate (figure 6).

2.5 Cyclophilin D can repress apoptosis induced by ANT-1

Our experiments with ANT-2 and cell death in yeast suggested that ANT-1's apoptotic activity is mediated by specific protein-protein interactions rather than by forming an active PT pore on its own. To check whether ANT-1 expression nevertheless activates the endogenous PT pore, we made use of bongkrekic acid, a specific inhibitor for the PT pore (Klingenberg et al., 1970). 50 μM of this compound repressed ANT-1 induced apoptosis in a quantitative apoptosis assay more than 2-fold (figure 7A). We therefore speculated that ANT-1 might titrate out some components of the endogenous PT pore and that replenishing these proteins might repress apoptosis. Consequently, we cotransfected ANT-1 and an expression vector for cyclophilin D, a component of the PT pore on the matrix side which directly interacts with ANT-1 (Woodfield et al., 1998). Quantification of apoptosis induction revealed a more than 2-fold reduced cell death by ANT-1 when cyclophilin D was cotransfected (figure 7B).

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Yao et al., 1996. J.Mol.Cell.Cardiol. 28: 95-101.

Zamzami et al., 1995a. J.Exp.Med. 182: 367-377.

Zamzami et al., 1995b. J.Exp.Med.181: 1661-1672.

Zamzami et al., 1998. Oncogene. 16: 1055-1063.

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Zoratti and Szabo, 1995. Biochim.Biophys.Acta. 1241: 139-176.

Claims

1. A method for the inhibition of apoptosis, comprising contacting a cell with an effective amount of a substance capable of inhibiting the activity of adenine nucleotide translocase-1 (ANT-1).

2. The method of claim 1, wherein said cell is a mammalian cell.

3. The method of claim 2, wherein said cell is associated with a pathogenic disorder.

4. The method of claim 1, wherein the activity of ANT-1 is inhibited on the nucleic acid level.

5. The method of claim 4, wherein the inhibition is effected by reducing ANT-1 gene expression.

6. The method of claim 4, wherein the activity of the endogenous ANT-1 promoter is reduced.

7. The method of claim 1, wherein the activity of ANT-1 is inhibited on the protein level.

8. The method of claim 7, wherein the inhibition is effected by adding ANT-1 protein antagonists.

9. The method of claim 8, wherein the antagonist is cyclophilin D.

10. The method of claim 1, wherein an apoptosis-inducing signal transduction pathway is inhibited, said pathway being activated by ANT-1.

11. A method for the treatment of diseases associated with excessive apoptosis, comprising the step of administering to a subject in need thereof a pharmaceutically effective amount of a substance capable of inhibiting the activity of adenine nucleotide translocase (ANT-1).

12. The method of claim 11, wherein the disease is a degenerative disease.

13. The method of claim 12, wherein the disease is dilated cardiomyopathy.

14. A method for identifying substances suitable for apoptosis inhibition comprising the step of determining the capability of a test substance to inhibit the activity of ANT-1.

15. The method of claim 14, wherein the capability of a test substance to bind ANT-1 or a domain thereof is determined.

16. The method of claim 14, wherein the capability of a test substance to bind the N-terminal domain of ANT-1 is determined.

17. The method of claim 14, wherein the capability of a test substance to inhibit the binding of ANT-1 to natural binding partners thereof is determined.

18. The method of claim 14, which is carried out as a high-throughput assay.

19. The method of claim 18, comprising a parallel determination of at least 96 test compounds.

The method of claim 14, which is carried out as a cell-based assay.

21. The method of claim 20, which is carried out as an assay using ANT-1-containing cell fractions or ANT-1-containing whole cells.

22. The method of claim 14, which is carried out as a molecular-based assay using an isolated protein selected from ANT-1 or a domain thereof.

23. The method of claim 22, wherein a recombinant protein is used.

24. The method of claim 14, wherein the determining step comprises the measurement of apoptosis induction.

25. The method of claim 24, wherein the apoptosis induction is measured by a parameter selected from the group consisting of DNA fragmentation, caspase activation or characteristic alterations in cell morphology.

26. A pharmaceutical composition comprising as an active agent an inhibitor of ANT-1 activity, optionally together with pharmaceutically acceptable diluents, carriers or adjuvants.

27. The pharmaceutical composition of claim 26 for use in the treatment of diseases associated with excessive apoptosis.

28. The composition of claim 27 for use in the treatment of human diseases.

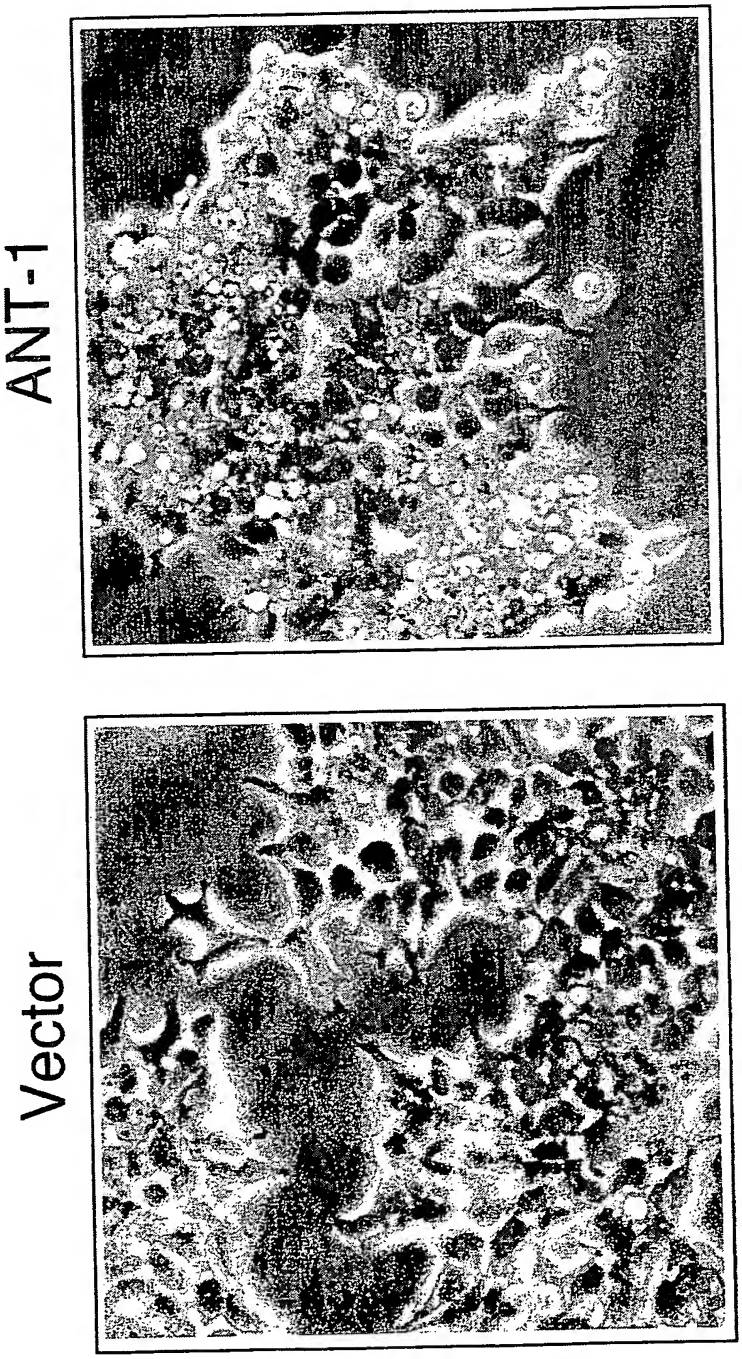
29. The composition of claim 28 for use in the treatment of dilated cardiomyopathy.

30. A method for the diagnosis of a degenerative disease or a predisposition therefor comprising detecting the ANT-1 expression in

a sample from tissue and/or body fluids of a subject to be tested, wherein elevated ANT-1 expression is indicative for a degenerative disease or a predisposition therefor.

31. The method of claim 30, wherein the degenerative disease is dilated cardiomyopathy.

Fig.1



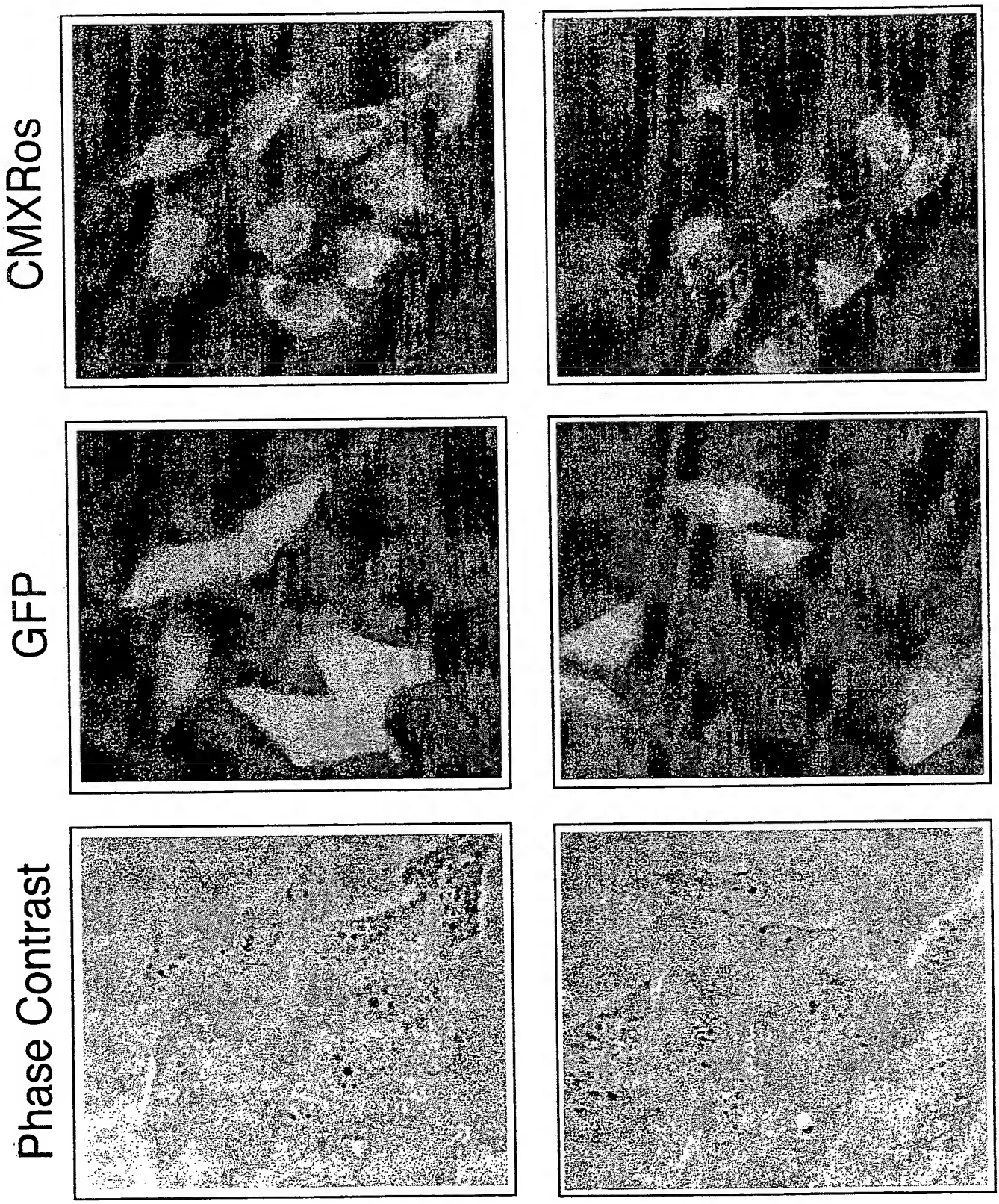


Fig. 2A

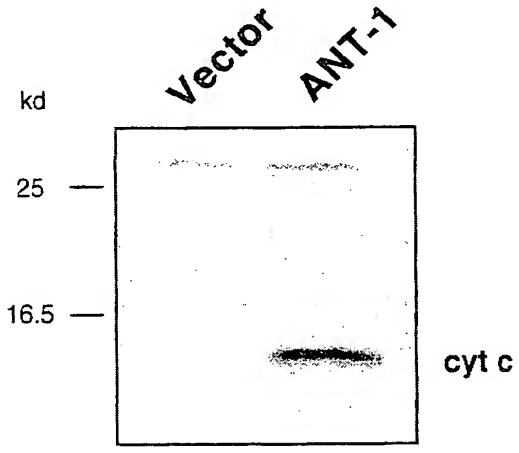
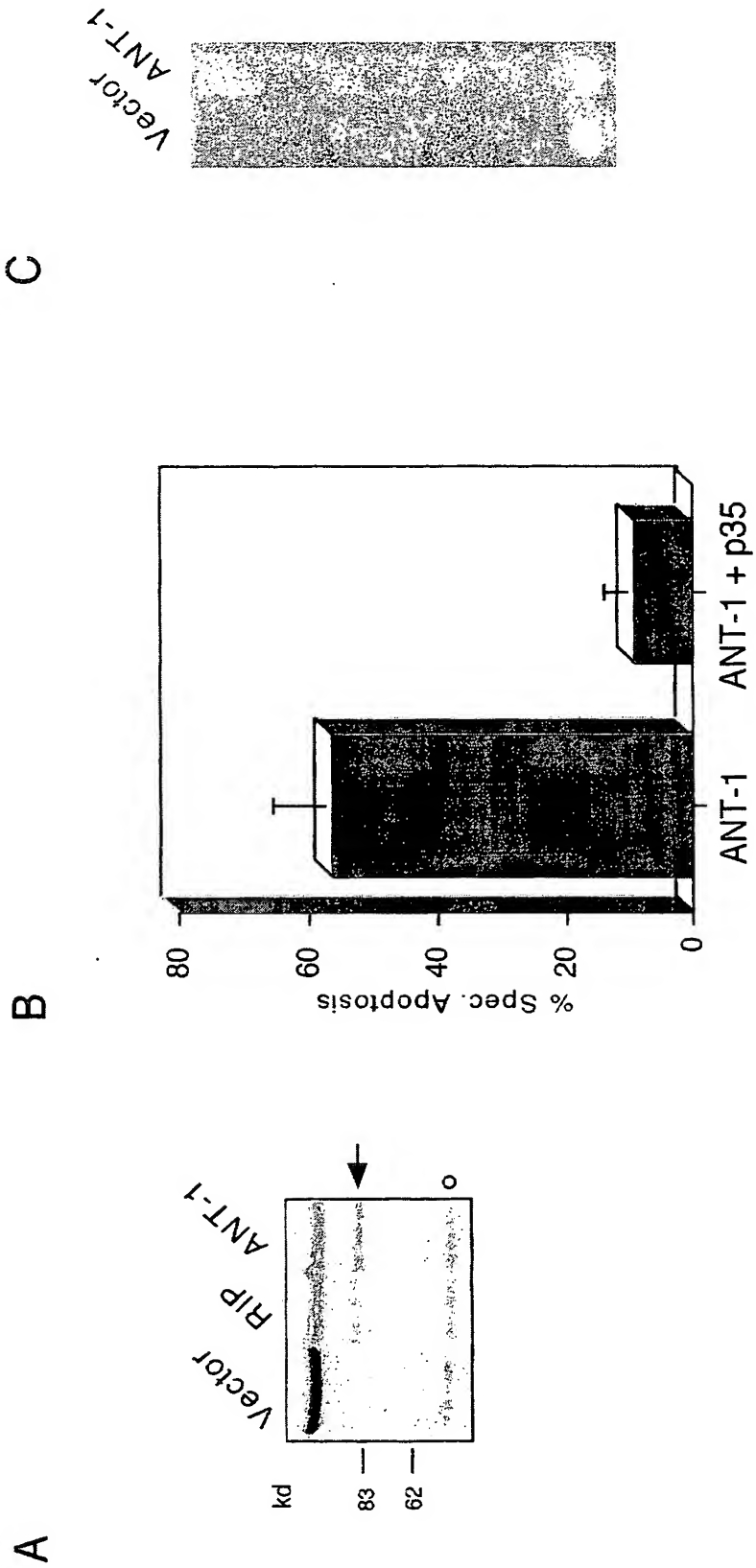


Fig. 2B

Fig. 3


C
Vector
ANT-1
B
% Spec. Apoptosis
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20
40
60
80
ANT-1
ANT-1 + p35
A
Vector
RIP
ANT-1
o
kd
83
62

Fig. 4

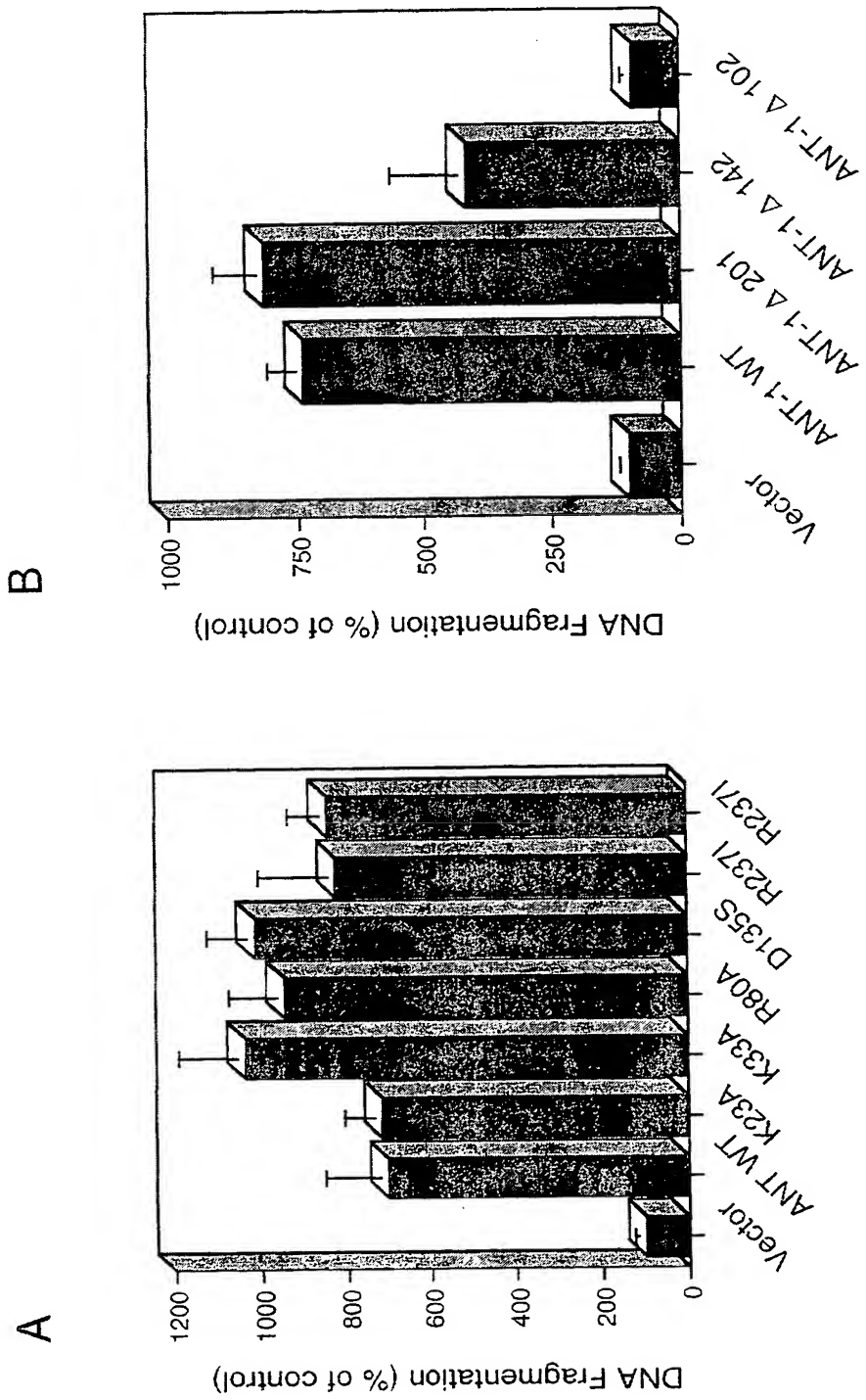
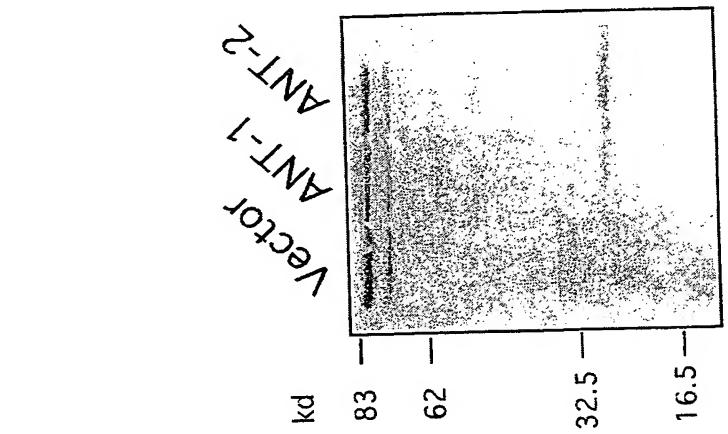

B
DNA Fragmentation (% of control)
0
250
500
750
1000
Vector
ANT-1 WT
ANT-1 Δ 201
ANT-1 Δ 142
ANT-1 Δ 102
A
DNA Fragmentation (% of control)
0
200
400
600
800
1000
1200
Vector
ANT WT
K23A
K33A
R80A
D135S
R237I
R237I

Fig. 5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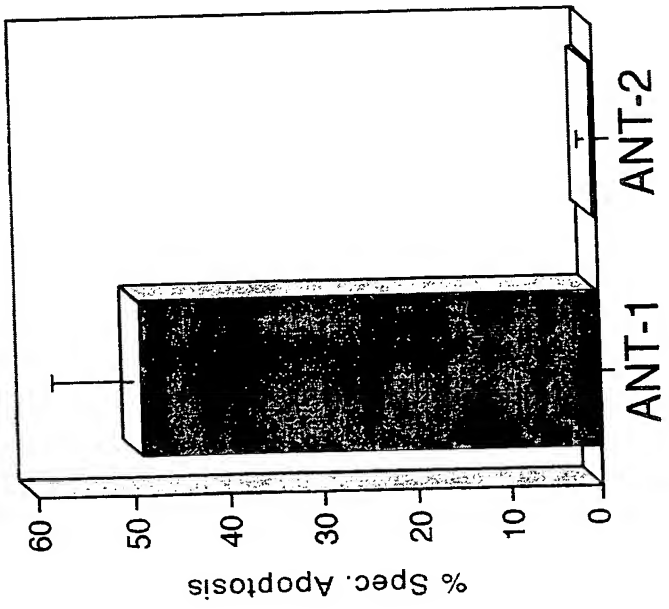
ANT-1
ANT-2
kd
83
62
32.5
16.5

B


% Spec. Apoptosis
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50
40
30
20
10
0
ANT-1
ANT-2

A

Fig. 6

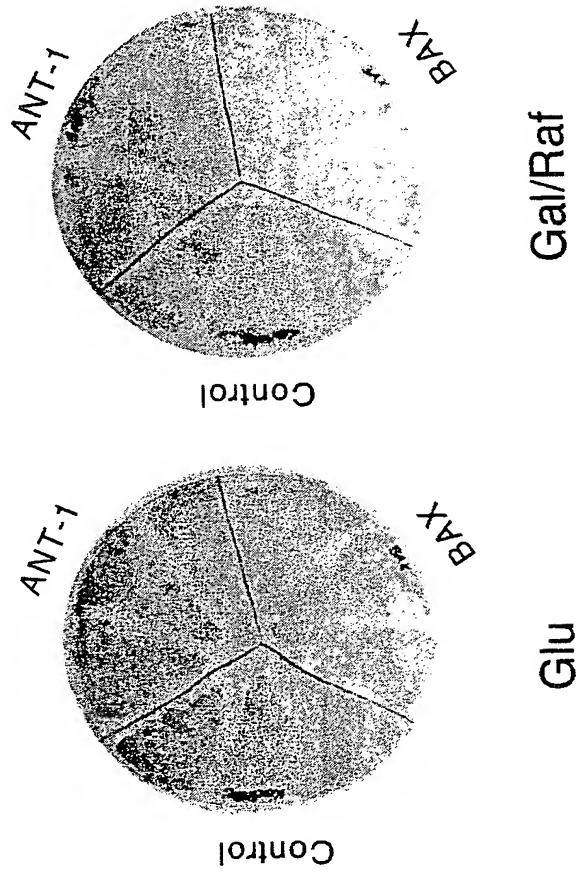
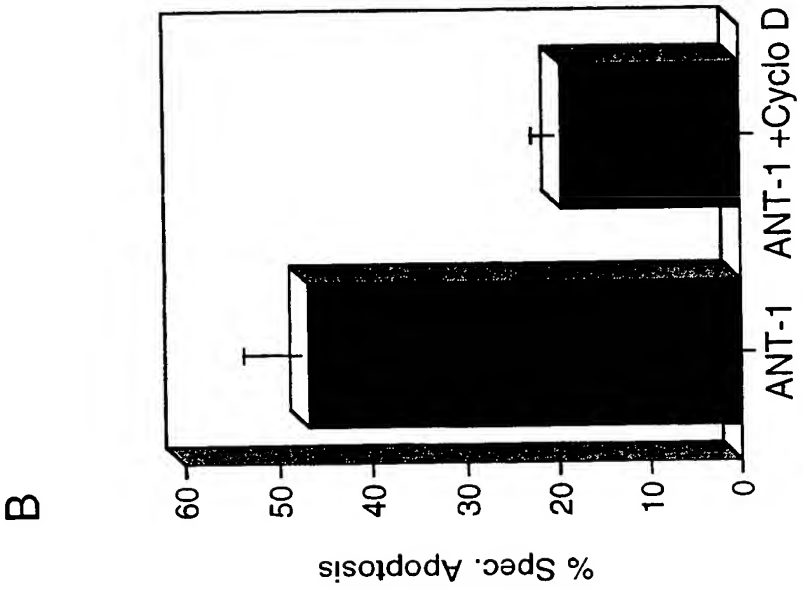
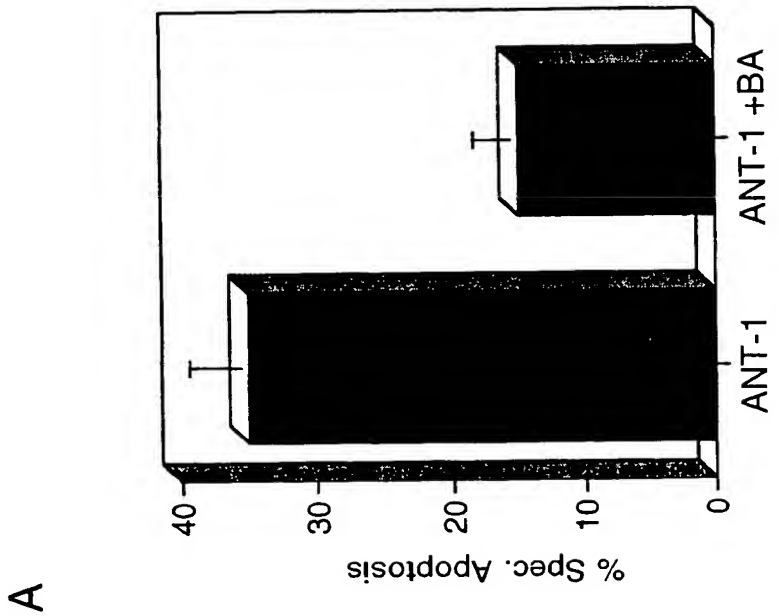


Fig. 7


B
% Spec. Apoptosis
0
10
20
30
40
50
60
ANT-1
ANT-1 +Cyclo D


A
% Spec. Apoptosis
0
10
20
30
40
ANT-1
ANT-1 +BA

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/08812

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/17 A61K38/52 G01N33/50 A61P9/00 /C12N15/52

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, MEDLINE, LIFESCIENCES, CHEM ABS Data, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DOERNER A ET AL: "Adenine nucleotide translocator in dilated cardiomyopathy: Pathophysiological alterations in expression and function." MOLECULAR AND CELLULAR BIOCHEMISTRY, vol. 174, no. 1-2, 1997, pages 261-269, XP000990253 the whole document ---	30,31
X	SYLVEN C ET AL: "Ventricular adenine nucleotide translocator mRNA is upregulated in dilate cardiomyopathy." CARDIOVASCULAR RESEARCH, vol. 27, no. 7, July 1993 (1993-07), pages 1295-1299, XP000990203 the whole document ---	30,31
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 March 2001	26/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Teyssier, B

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HALESTRAP A P ET AL: "Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart." BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1366, no. 1-2, 10 August 1998 (1998-08-10), pages 79-94, XP000982881	
A	KHASPEKOV L ET AL: "Cyclosporin A and its nonimmunosuppressive analogue N-Me-Val-4-cyclosporin A mitigate glucose/oxygen deprivation-induced damage to rat cultured hippocampal neurons." EUROPEAN JOURNAL OF NEUROSCIENCE, vol. 11, no. 9, 1999, pages 3194-3198, XP000982879	
P,X	WO 00 26370 A (ANDERSON CHRISTEN M ;CLEVENGER WILLIAM (US); WILEY SANDRA EILEEN () 11 May 2000 (2000-05-11) page 44 -page 58; examples 12-14	14-29
P,X	BAUER M K A ET AL: "Adenine nucleotide translocase-1, a component of the permeability transition pore, can dominantly induce apoptosis." JOURNAL OF CELL BIOLOGY, vol. 147, no. 7, 27 December 1999 (1999-12-27), pages 1493-1501, XP000990027 the whole document	1-31

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 00/08812

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0026370 A	11-05-2000	AU 2472900 A	22-05-2000
		EP 1049780 A	08-11-2000

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 March 2001 (22.03.2001)

PCT

(10) International Publication Number
WO 01/19384 A3

(51) International Patent Classification[7]: A61K 38/17, 38/52, G01N 33/50, A61P 9/00 // C12N 15/52

(21) International Application Number: PCT/EP00/08812

(22) International Filing Date:
8 September 2000 (08.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/153,040 10 September 1999 (10.09.1999) US

(71) Applicant *(for all designated States except US)*: **MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN E.V.** [DE/DE]; Hofgartenstrasse 8, 80539 München (DE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **GRIMM, Stefan** [DE/DE]; Richard-Riemerschmid-Allee 7, 81241 München (DE). **BAUER, Manuel** [DE/DE]; Stöberlstr. 50, 80686 München (DE). **SCHUBERT, Alexis** [DE/DE]; Clemensstr. 74, 80796 München (DE). **CRAMER, Ursula** [DE/DE]; Gräfstr. 28, 81241 München (DE).

(74) Agents: **WEICKMANN, H.** et al.; Kopernikusstrasse 9, 81679 München (DE).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(88) Date of publication of the international search report:
27 September 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/19384 A3

(54) Title: ANT-1 AS DRUG TARGET

(57) Abstract: The present invention refers to the use of adenine nucleotide translocase-1 (ANT-1), a central component of the permeability transition pore in mitochondria, as a drug target, particularly for the treatment of dilated cardiomyopathy. Further, an assay for the detection of pharmacologically active substances is disclosed which inhibit ANT-1 activity. In addition, ANT-1 expression is claimed as a diagnostic marker for dilated cardiomyopathy.

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
20985P WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/08812	08/09/2000	10/09/1999
International Patent Classification (IPC) or national classification and IPC A61K38/00		
Applicant MAX-PLANCK-GESELLSCHAFT ZUR FORDERUNG DER WISS...		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
04/04/2001	02.11.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Fayos, C Telephone No. +49 89 2399 2180

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/08812

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-17	as originally filed				

Claims, No.:

1-31	as received on	17/10/2001	with letter of	17/10/2001	

Drawings, sheets:

1/8-8/8	as originally filed				

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 1-13 (industrial applicability).

because:

☒ the said international application, or the said claims Nos. 1-13 (industrial applicability) relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion

could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-31
	No:	Claims	-
Inventive step (IS)	Yes:	Claims	1-31
	No:	Claims	-
Industrial applicability (IA)	Yes:	Claims	1-13 (see separate sheet); 14-31
	No:	Claims	-

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item II

Priority

1- The current assessment is based on the assumption that all claims enjoy priority rights from the filing date of the priority document. It the later turns out that is not correct, the documents D5 and D6 cited in the international search report could become relevant.

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

2- Claims 1-13 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

3- Reference is made to the following documents:

D1: DOERNER A ET AL: 'Adenine nucleotide translocator in dilated cardiomyopathy: Pathophysiological alterations in expression and function.' MOLECULAR AND CELLULAR BIOCHEMISTRY, vol. 174, no. 1-2, 1997, pages 261-269, XP000990253

D2: SYLVEN C ET AL: 'Ventricular adenine nucleotide translocator mRNA is upregulated in dilate cardiomyopathy.' CARDIOVASCULAR RESEARCH, vol. 27, no. 7, July 1993 (1993-07), pages 1295-1299, XP000990203

D3: HALESTRAP A P ET AL: 'Elucidating the molecular mechanism of the permeability transition pore and its role in reperfusion injury of the heart.' BIOCHIMICA ET BIOPHYSICA ACTA, vol. 1366, no. 1-2, 10 August 1998 (1998-08-10), pages 79-94, XP000982881

D4: KHASPEKOV L ET AL: 'Cyclosporin A and its nonimmunosuppressive analogue N-

Me-Val-4-cyclosporin A mitigate glucose/oxygen deprivation-induced damage to rat cultured hippocampal neurons.' EUROPEAN JOURNAL OF NEUROSCIENCE, vol. 11, no. 9, 1999, pages 3194-3198, XP000982879

D5: see item VI

D6: BAUER M K A ET AL: 'Adenine nucleotide translocase-1, a component of the permeability transition pore, can dominantly induce apoptosis.' JOURNAL OF CELL BIOLOGY, vol. 147, no. 7, 27 December 1999 (1999-12-27), pages 1493-1501, XP000990027

NOVELTY - Art. 33 (1) and (2) PCT

4- Claims 1-31 appear to be novel in the light of the prior art cited in the search report.

4.1- The novel features are:

a pharmaceutical composition comprising as an active agent an inhibitor of ANT-1 activity, and

a method for the diagnosis of a degenerative disease or a predisposition therefor comprising detecting the ANT-1 expression in a sample from tissue and / or body fluids of a subject to be tested.

INVENTIVE STEP - Art. 33 (1) and (3) PCT

5- Claims 1-31 appear to be inventive in the light of the cited prior art.

5.1- D1 and / or D2 show that ANT-1 expression is increased in dilated cardiomyopathy. However, neither D1, nor D2 suggest the association of an elevated ANT-1 expression with an apoptotic process in degenerative diseases.

INDUSTRIAL APPLICABILITY - Art. 33 (1) and (4) PCT

6- For the assessment of the present claims 1-13 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The

patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VI
Certain documents cited

7- Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
D5: WO 00 26370	11.05.00	03.11.99	03.11.98 08.09.99

Re Item VIII
Certain observations on the international application

8- Claims 1-8, 10 and 11-13 do not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The claims attempt to define the subject-matter in terms of the result to be achieved ("substance capable of inhibiting the activity of ANT-1") which merely amounts to a statement of the underlying problem (inhibiting the activity of ANT-1"). The technical features necessary for achieving this result are missing.

9- Furthermore, claims 1-8, 10 and 11-13 are not supported by the technical contents of the description as required by Article 6 PCT, as their scope is broader than justified by the description (use of cyclophilin-D).

Claims

1. A method for the inhibition of apoptosis, comprising contacting a cell with an effective amount of a substance capable of inhibiting the activity of adenine nucleotide translocase-1 (ANT-1).

2. The method of claim 1, wherein said cell is a mammalian cell.

3. The method of claim 2, wherein said cell is associated with a pathogenic disorder.

4. The method of claim 1, wherein the activity of ANT-1 is inhibited on the nucleic acid level.

5. The method of claim 4, wherein the inhibition is effected by reducing ANT-1 gene expression.

6. The method of claim 4, wherein the activity of the endogenous ANT-1 promoter is reduced.

7. The method of claim 1, wherein the activity of ANT-1 is inhibited on the protein level.

8. The method of claim 7, wherein the inhibition is effected by adding ANT-1 protein antagonists.

9. The method of claim 8, wherein the antagonist is cyclophilin D.

10. The method of claim 1, wherein an apoptosis-inducing signal transduction pathway is inhibited, said pathway being activated by ANT-1.

11. A method for the treatment of diseases associated with excessive

apoptosis, comprising the step of administering to a subject in need thereof a pharmaceutically effective amount of a substance capable of inhibiting the activity of adenine nucleotide translocase (ANT-1).

12. The method of claim 11, wherein the disease is a degenerative disease.

13. The method of claim 12, wherein the disease is dilated cardiomyopathy.

14. A method for identifying substances suitable for apoptosis inhibition comprising the step of determining the capability of a test substance to inhibit the activity of ANT-1.

15. The method of claim 14, wherein the capability of a test substance to bind ANT-1 or a domain thereof is determined.

16. The method of claim 14, wherein the capability of a test substance to bind the N-terminal domain of ANT-1 is determined.

17. The method of claim 14, wherein the capability of a test substance to inhibit the binding of ANT-1 to natural binding partners thereof is determined.

18. The method of claim 14, which is carried out as a high-throughput assay.

19. The method of claim 18, comprising a parallel determination of at least 96 test compounds.

20. The method of claim 14, which is carried out as a cell-based assay.

21. The method of claim 20, which is carried out as an assay using ANT-1-containing cell fractions or ANT-1-containing whole cells.

22. The method of claim 14, which is carried out as a molecular-based assay using an isolated protein selected from ANT-1 or a domain thereof.

23. The method of claim 22, wherein a recombinant protein is used.

24. The method of claim 14, wherein the determining step comprises the measurement of apoptosis induction.

25. The method of claim 24, wherein the apoptosis induction is measured by a parameter selected from the group consisting of DNA fragmentation, caspase activation or characteristic alterations in cell morphology.

26. A pharmaceutical composition comprising as an active agent an inhibitor of ANT-1 activity, optionally together with pharmaceutically acceptable diluents, carriers or adjuvants.

27. The pharmaceutical composition of claim 26 for use in the treatment of diseases associated with excessive apoptosis.

28. The composition of claim 27 for use in the treatment of human diseases.

29. The composition of claim 28 for use in the treatment of dilated cardiomyopathy.

30. A method for the diagnosis of an apoptotic process in a degenerative disease or a predisposition therefor comprising detecting the ANT-1 expression in a sample from tissue and/or body fluids of a subject to be tested, wherein elevated ANT-1 expression is indicative for an apoptotic process occuring in a degenerative disease or a predisposition therefor.

31. The method of claim 30, wherein the degenerative disease is dilated cardiomyopathy.

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 22 May 2001 (22.05.01)	
International application No. PCT/EP00/08812	**Applicant's or agent's file reference** 20985P WO
International filing date (day/month/year) 08 September 2000 (08.09.00)	**Priority date** (day/month/year) 10 September 1999 (10.09.99)
Applicant GRIMM, Stefan et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

04 April 2001 (04.04.01)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Claudio Borton
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

WEICKMANN WEICKMANN HUBER LISKA
PRECHTEL BÖHM WEISS TIESMEYER
HERZOG RUTTENSPERGER JORDAN
Kopernikusstrasse 9
D-81679 München
ALLEMAGNE

PCT

6. NOV. 2001

Eing. Patentanwälte

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	02.11.2001
Applicant's or agent's file reference: 20985P WO	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/08812	08/09/2000	10/09/1999

Applicant: MAX-PLANCK-GESELLSCHAFT ZUR FORDERUNG DER WISS...

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Ferro Vasconcelos, M Tel.+49 89 2399-~~7995~~ 8042

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION**	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
20985P WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/ 08812	08/09/2000	10/09/1999
Applicant		
MAX-PLANCK-GESELLSCHAFT ZUR FORDERUNG DER WISS...		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.